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for PD Therapy

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In PD the insult to the dopamine (DA) neuron is posited to involve oxidative injury mediated by mitochondrial respiratory abnormalities and through participation of oxidative adducts made onto DA and presynaptic target proteins such as  $\alpha$ -synuclein. The misfolding of  $\alpha$ -synuclein engendered by oxidative adduct formation is hypothesized to be a critical participating process in Lewy Body formation and dopamine neuron compromise and death. *Our central hypothesis purports that protein aggregates forming within dopaminergic neurons are seeded and require misfolded  $\alpha$ -synuclein and that these aggregates are cytotoxic thereby contributing directly to neuron death.* Thus targeting  $\alpha$ -synuclein protein misfolding will enable the development of effective therapy. The main goal of this application is to identify and characterize humanized single chain antibodies (scFvs) that recognize structural epitopes on  $\alpha$ -synuclein and utilize these scFvs to attenuate the pathology associated with  $\alpha$ -synuclein misfolding. Thusfar we have expressed, purified and generated altered conformers of  $\alpha$ -synuclein; screened for, identified and purified synuclein conformer-specific scFvs. We have begun the characterization of these synuclein-specific scFvs in tissue culture and animal models of  $\alpha$ -synuclein overexpression.

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## INTRODUCTION

Parkinson's disease (PD) is clinical syndrome triggered by disparate mechanisms. The clinical and neuropathological features are indistinguishable among the mechanistically different forms thus suggesting a convergent shared pathway. The disease invariably produces loss of dopamine neurons in the substantia nigra (SN). The selective insult to the dopamine (DA) neuron is posited to involve oxidative injury mediated, in part, by mitochondrial respiratory abnormalities and through participation of oxidative adducts made onto DA and presynaptic target proteins such as  $\alpha$ -synuclein. Convergence in PD pathogenesis has been greatly aided by the linkage between mitochondrial toxicant (e.g., MPTP, paraquat, rotenone) injury, inherited defects in turnover of the presynaptic and Lewy Body constituent protein  $\alpha$ -synuclein, and involvement of cytosolic DA itself. Recent data directly implicate DA-quinone in the modification of  $\alpha$ -synuclein and its inducement of misfolding ( $\beta$ -sheet conformation) and aggregation. ***Taken together these data support our central hypothesis that protein aggregates forming within dopaminergic neurons are seeded and require misfolded  $\alpha$ -synuclein and that these aggregates are cytotoxic thereby contributing directly to neuron death.*** We propose that either interference with aggregate formation or dissolution of existing aggregates will attenuate the pathophysiology of PD. To this end, we employ an approach to rapidly identify humanized single chain antibodies (scFvs) that recognize structural epitopes on candidate pathogenic proteins. These scFvs bind pathogenic structural conformers with submicromolar affinity, can be engineered for secretion or cytosolic localization (intrabodies), and configured for high-level gene expression from viral vectors developed in my laboratory.

## BODY

Our progress on this project is described below. We have divided it into 6 sections (I.A., I.B., I.D., II.A., II.B. and III.A.) to follow our original Specific Aims. In addition we have included the Statement of Work as it applies to Year 02.

## STATEMENT OF WORK

In PD the insult to the dopamine (DA) neuron is posited to involve oxidative injury mediated by mitochondrial respiratory abnormalities and through participation of oxidative adducts made onto DA and presynaptic target proteins such as  $\alpha$ -synuclein ( $\alpha$ -syn). The misfolding of  $\alpha$ -syn engendered by oxidative adduct formation is hypothesized to be a critical participating process in Lewy Body formation and dopamine neuron compromise and death. Thus targeting  $\alpha$ -syn protein misfolding will enable the development of effective therapy. **The main goals of this application for Year 02 is to assess the efficacy of  $\alpha$ -synuclein scFv in the attenuation of cellular stress, to determine and identify humanized single chain antibodies (scFvs) that recognize different conformers of this protein.** In years 02-05 we propose to identify the structural epitopes on  $\alpha$ -syn that interact with the scFvs and utilize these scFvs to attenuate the pathology associated with  $\alpha$ -syn misfolding.

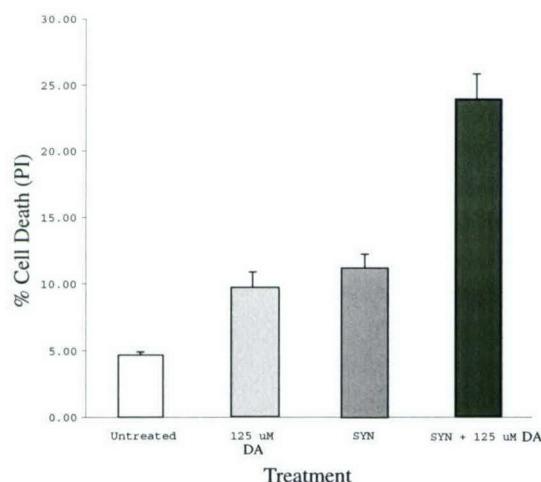


## PROGRESS

### I.A. Expression of wildtype and mutant $\alpha$ -synuclein.

**Progress:** Doxycycline regulatable cell lines expressing  $\alpha$ -synuclein have been further characterized. Overexpression of  $\alpha$ -synuclein in the presence of dopamine results in increased cell death in a dopaminergic-like cell line.

Upon further characterization of  $\alpha$ -synMN9D cell lines we have determined that overexpression of  $\alpha$ -synuclein in these dopaminergic-like cells is toxic (**Figure 1**). Furthermore, this toxicity is exacerbated in the presence of dopamine (**Figure 1**). This and previous data from our laboratory suggests that the conformation of  $\alpha$ -synuclein is altered in the presence of dopamine and this change results in increased cell death. This system will be utilized to test the efficacy of our newly identified conformer-specific scFvs.



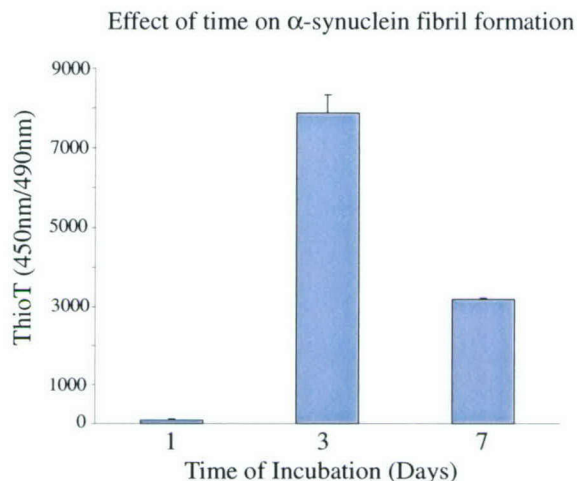
**Figure 1. Effect of SYN and dopamine on cell viability.** Regulatable  $\alpha$ SYNMN9D cells were grown in the absence (untreated) and presence (SYN) of doxycycline to induce  $\alpha$ -synuclein expression. Cells were also incubated with either vehicle or 125  $\mu$ M dopamine. Cell death measurements were conducted using propidium iodide cell sorting techniques.

### I.B. Generation of altered conformers.

**Progress:** Altered conformers of wildtype, A53T and A30P  $\alpha$ -synuclein have been characterized by western blot analysis, AFM, denaturing PAG electrophoresis, thioT binding and EM.

Altered  $\alpha$ -synuclein conformers have been produced *in vitro* and characterized by thioflavin T assays. Previously we have identified altered conformers by gel analysis and AFM. The thioflavin T assay allows us to quantitate fibril formation in the  $\alpha$ -synuclein samples. As shown in **Figure 2**, we demonstrate that the maximum fibril formation occurs at day 3. Interestingly by Day 7 the fibril formation decreases when compared to Day 3. Likewise utilizing denaturing polyacrylamide gel electrophoresis followed by protein staining with Simply Blue, we have demonstrated an increase in large molecular weight protein aggregates at the 7 day time point compared to the 3 day time point (data not shown). This data suggests a movement from fibrils to aggregates with a net loss in

fibril content over time. We are currently investigating the toxicity of both the fibrillar and aggregate forms of  $\alpha$ -synuclein. This data combined with the data in I.A. further supports the hypothesis that changes in  $\alpha$ -synuclein conformation occur in the presence of dopamine and result in cell death.

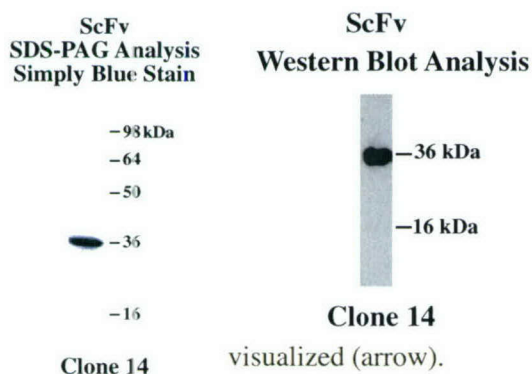


**Figure 2. Thioflavin T assay to determine fibril formation.** Bacterially expressed and purified  $\alpha$ -synuclein was incubated at 33°C/1,000rpm for 1-7 days and fibril formation determined by ThioT assay.

#### **I.D. Characterization of anti-syn single chain antibodies.**

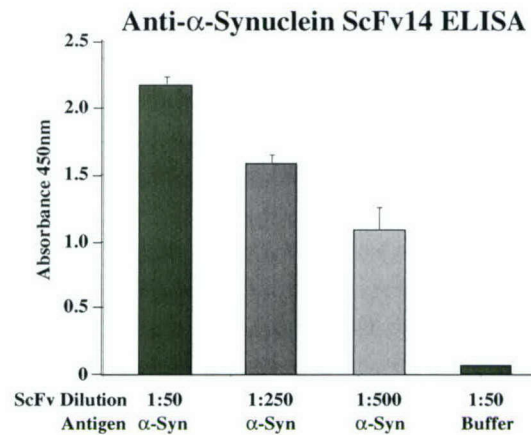
**Progress:** Single-chain antibodies with specificity for different  $\alpha$ -synuclein conformers (monomer, aggregated and dopamine adducted) have been identified, expressed and purified. ScFv14 was shown to bind specifically to one of twenty-five overlapping wild-type  $\alpha$ -synuclein linear peptides.

**ScFv purification and  $\alpha$ -synuclein reactivity:** Our scFvs are subcloned into the pComb vector which append several sequences that facilitate both purification (6x HIS) and detection (HA). For example, bacterially expressed scFv14 was isolated and purified using Talon resin technology. The purity of scFv14 was verified by coomassie blue staining following denaturing polyacrylamide gel (SDS-PAGE) electrophoresis as well as by western blot analysis (**Figure 3**). Furthermore, purified scFv14 binds to  $\alpha$ -synuclein in a dose-dependent fashion (**Figure 4**). We have purified and characterized several other  $\alpha$ -synuclein scFvs (see **Table 1**).



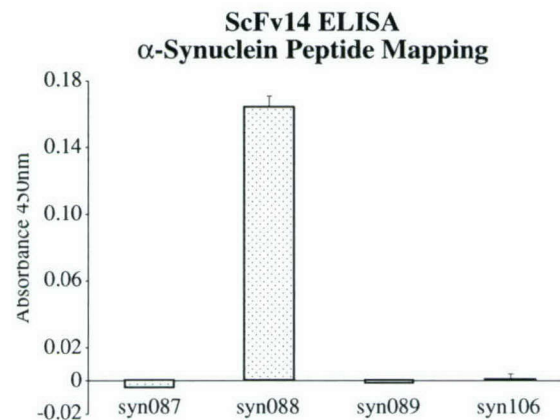
**Figure 3. ScFv Purity.** ScFv14 was expressed and purified from bacterial cultures. **Left Panel.** 1  $\mu$ g of purified protein was subjected to SDS-PAGE electrophoresis and stained with Simply Blue stain. **Right Panel.** 1  $\mu$ g of purified protein was subjected to SDS-PAGE electrophoresis and western blot analysis with anti-HA HRP antibodies. In both panels one major band corresponding to ScFv14 is





**Figure 4. Anti- $\alpha$ -synuclein ScFv14 ELISA.** Bacterially expressed and purified ScFv14 was diluted and tested in an  $\alpha$ -syn ELISA. The binding of ScFv was antibody concentration-dependent. Wells coated with buffer instead of  $\alpha$ -syn served as the negative control.

**Conformer specificity:** We have generated a permuted panel of  $\alpha$ -syn peptides spanning the entire polypeptide and have tested anti-syn scFv14 against this panel to identify which linear epitopes are recognized. Of the 25 peptides tested, scFv14 recognized only one linear peptide, syn088 (**Figure 5**).



**Figure 5. ScFv14 ELISA: $\alpha$ -synuclein peptide mapping.** Bacterially expressed and purified ScFv14 was tested in an ELISA designed to identify the linear peptide region of  $\alpha$ -syn responsible for ScFv interaction. Twenty-five 15 aa overlapping peptides corresponding to human  $\alpha$ -syn were utilized. Only 4 peptides are shown including 3 non-recognized peptides and the one recognized peptide, the remaining 20 peptides were not recognized. The peptides were biotin-linked and assayed on streptavidin-coated plates. ScFv14 binds the syn088 peptide corresponding to aa 106-120.

In addition, we have screened the human phage scFv library against the altered forms of  $\alpha$ -syn to identify conformation specific single-chain antibodies. We have identified 5 scFVs that demonstrate specificity for  $\alpha$ -syn aggregated in the presence of dopamine (Table 1). All scFv clones have been sequenced and contain different complementarity determining regions.

**Table 1.  $\alpha$ -Synuclein-Specific ScFvs**

ScFv clone #	Antigen panned against*	Antigen recognized**
14	$\alpha$ -syn monomer	$\alpha$ -syn monomer
15	$\alpha$ -syn monomer	$\alpha$ -syn monomer
3	$\alpha$ -syn:DAQ	$\alpha$ -syn monomer, $\alpha$ -syn aggregates, SYN:DAQ
4	$\alpha$ -syn:DAQ	SYN:DAQ, BSA:DAQ
5	$\alpha$ -syn:DAQ	$\alpha$ -syn monomer, $\alpha$ -syn aggregates, SYN:DAQ, BSA:DAQ
6	$\alpha$ -syn:DAQ	SYN:DAQ, BSA:DAQ
7	$\alpha$ -syn:DAQ	SYN:DAQ, BSA:DAQ
8	$\alpha$ -syn:DAQ	SYN:DAQ, BSA:DAQ
10	$\alpha$ -syn:DAQ	SYN:DAQ, BSA:DAQ

\*Antigen panned against: a human scFv library was panned against different conformers of  $\alpha$ -synuclein and phage identified. \*\*Antigen recognized: following initial panning scFvs were expressed, purified and tested against the full panel of  $\alpha$ -synuclein conformers.

In conclusion, we have identified, purified and begun to characterize 9 scFv's with differentially binding to various  $\alpha$ -synuclein conformers.

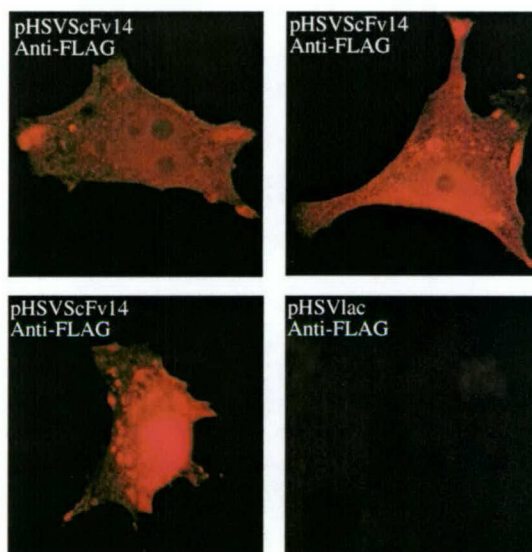
#### **II.A. Attenuation of $\alpha$ -syn induced toxicity by conformer-specific scFvs.**

**Progress:** The identified single-chain antibodies have been subcloned into HSV amplicon and rAAV shuttle vectors for virus vector-mediated intrabody expression. ScFv14 intrabodies have been detected in a mammalian cell line following transfection with pHSVscFv14.

The identified scFvs have been subcloned into an HSV amplicon plasmid backbone vector for intrabody expression. This vector contains the necessary DNA sequences for efficient viral packaging (ori"S"; packaging "a" site); the HSV IE4/5 promoter followed by a multicloning region for insertion of the structure specific scFv (HSVscFv). The hemagglutinin epitope sequence (HA tag) has been fused to the C termini and the FLAG epitope sequence has been fused to the N termini of all scFv constructs to facilitate immunolocalization and immunoprecipitation. **Figure 6** demonstrates that HSVscFv constructs can be expressed in mammalian cells. HSVscFv amplicon virus is currently under production. ScFvs have also been subcloned into a rAAV expression construct for our planned animal studies to ensure long-term scFv expression.



ScFv14 Intrabody Expression  
Anti-FLAG ICC  
following pHSVScFv14 transfection of HN33.11 cells



**Figure 6. ScFv14 Intrabody Expression: Anti-FLAG ICC.** HN33.11 cells were transfected with either pHSVScFv14 or pHSVlac. Cells were fixed 48 hrs. later and ScFv14 detected by anti-FLAG fluorescent immunocytochemistry. Intrabodies were detected in pHSVScFv14 transfected cells only.

In conclusion, scFv's have been developed which are capable of intracellular expression in mammalian cells. These intrabodies are currently being characterized with regard to  $\alpha$ -synuclein induced toxicity.

**II.B. Conformation specific scFv attenuation of toxicity induced by  $\alpha$ -syn combined with oxidative stress.**

**Progress:** MN9D $\alpha$ synwt cells demonstrate increased cell death in the presence of dopamine. The effect of dopamine and paraquat is currently under investigation.

As shown in **Figure 1**, there is an increase in MN9D cell death when both dopamine and synuclein are present. We are currently testing the effect of paraquat on the MN9D $\alpha$ synwt cells in the presence of dopamine. Once these oxidative stress conditions are established we will test the effect of our conformation-specific scFvs on cell death in  $\alpha$ -syn overexpressing dopaminergic-like cells.

**III.A. Primary protection of scFvs administered concurrently with subchronic paraquat and MPTP to hm2  $\alpha$ -syn transgenic mice.**

**Progress:** A cohort of transgenic mice which overexpress  $\alpha$ -synuclein (hm2 $\alpha$ syn) have been treated with paraquat and initial characterization begun.

To establish the low paraquat dose paradigm we have injected 6 hm2  $\alpha$ -syn transgenic and 6 non-transgenic mice with either paraquat (2.0mg/kg) or saline, once a week for 24 weeks. Mice were sacrificed one week after the last injection with half of the brains from each group microdissected for neurochemistry and protein analysis. The remaining animals were fixed via cardiac perfusion with 4% paraformaldehyde and brains sectioned using a sliding microtome. Brain sections are being utilized for  $\alpha$ -synuclein and tyrosine

hydroxylase IHC to enumerate TH+ cells as well as to changes in neurites and  $\alpha$ -synuclein accumulation. Similarly, substantia nigra sections are currently being examined for the content of  $\alpha$ -synuclein and TH+ neurons enumerated. Hm2 $\alpha$ -syn mice will be compared to non-transgenic control mice.

### **KEY RESEARCH ACCOMPLISHMENTS**

- Doxycycline regulatable cell lines expressing  $\alpha$ -synuclein have been further characterized.
- Overexpression of  $\alpha$ -synuclein in the presence of dopamine results in increased cell death in a dopaminergic-like cell line.
- Altered conformers of wildtype, A53T and A30P  $\alpha$ -synuclein have been characterized by western blot analysis, AFM, denaturing PAG electrophoresis, thioT binding and EM.
- Single-chain antibodies with specificity for different  $\alpha$ -synuclein conformers (monomer, aggregated and dopamine adducted) have been identified, expressed and purified.
- ScFv14 binds one of twenty-five linear peptides that span wild-type  $\alpha$ -synuclein .
- The identified single-chain antibodies have been subcloned into HSV amplicon and rAAV shuttle vectors for virus vector-mediated intrabody expression.
- ScFv14 intrabodies have been detected in a mammalian cell line following transfection with pHSVscFv14.
- A cohort of transgenic mice which overexpress  $\alpha$ -synuclein have been treated with paraquat.
- Further characterization of the neurotoxic insult in these mice is underway.

Future studies will include continued characterization of anti- $\alpha$ -syn scFv interactions with  $\alpha$ -synuclein *in vitro* and *in vivo*.

### **REPORTABLE OUTCOMES**

#### **Manuscripts**

**Maguire-Zeiss KA** and Federoff HJ. Novel Gene Therapeutic Strategies for Neurodegenerative Diseases. In: Opportunities and Challenges of the Therapies Targeting CNS Regeneration, Ernst Schering Research Foundation, in press, 2004.

#### **Abstracts**

**Maguire-Zeiss, KA**, Yehling, E, Giuliano R, Sullivan M, and Federoff HJ. American Society of Gene Therapy 7<sup>th</sup> Annual Meeting June 2-6, 2004. HSV Amplicon Expression of Single Chain Antibodies Directed Against  $\alpha$ -Synuclein Conformers.

### **CONCLUSIONS**

We hypothesize that in dopamine neurons a steady state exists between the native  $\alpha$ -synuclein, the  $\beta$ -sheet conformer and the aggregated form. Pushing the balance towards pathophysiological protein conformers will result in cell death. We propose that scFvs which specifically interact with monomer  $\alpha$ -syn will reduce the steady state concentrations of oligomers and mitigate toxicity. Thus targeting  $\alpha$ -synuclein protein



misfolding will enable the development of effective therapy. Toward this goal we have expressed, purified and produced altered  $\alpha$ -synuclein conformers for screening against a combinatorial phage display library expressing human immunoglobulin heavy and light chain variable regions. We have identified two scFvs that preferentially recognize monomeric  $\alpha$ -synuclein, five that recognize dopamine-adducted proteins (both  $\alpha$ -synuclein and BSA), one that recognizes dopamine-adducted  $\alpha$ -synuclein, as well as  $\alpha$ -synuclein monomers and aggregates but not dopamine-adducted BSA, and one with broad reactivity for monomer, aggregated and dopamine adducted  $\alpha$ -synuclein as well as dopamine-adducted BSA. We are now testing these scFvs in conditionally overexpressing  $\alpha$ -synuclein cell lines to determine whether they interfere with  $\alpha$ -synuclein aggregate formation and aid in the dissolution of existing aggregates. We have begun the proposed animal studies and are finishing the analysis of the paraquat paradigm. Subsequent years will extend these findings *in vivo* to animal models of PD treated with rAAVscFvs.

#### **REFERENCES**

N/A